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Review

Biochemical interactions in the wnt pathway

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Abstract

The wnt signal transduction pathway is involved in many differentiation events during embryonic development and can lead to tumor formation after aberrant activation of its components. The cytoplasmic component β-catenin is central to the transmission of wnt signals to the nucleus: in the absence of wnts β-catenin is constitutively degraded in proteasomes, whereas in the presence of wnts β-catenin is stabilized and associates with HMG box transcription factors of the LEF/TCF family. In tumors, β-catenin degradation is blocked by mutations of the tumor suppressor gene APC (adenomatous polyposis coli), or of β-catenin itself. As a consequence, constitutive TCF/β-catenin complexes are formed and activate oncogenic target genes. This review discusses the mechanisms that silence the pathway in cells that do not receive a wnt signal and goes on to describe the regulatory steps involved in the activation of the pathway.

Keywords: Wnt signal transduction; Tumor development; Adenomatous polyposis coli; β-Catenin; TCF family; Gene expression

1. Introduction

1.1. Overview of the wnt pathway

Wnts constitute a family of secreted glycoproteins with distinct expression patterns in the embryo and in the adult organism. Wnts appear to be involved in differentiation processes by controlling embryonic induction, polarity of cell division, cell fate and growth [1]. A role for wnts in tumorigenesis was first suggested by the identification of the mouse wnt-1 (int-1) (proto-)oncogene, which is frequently activated by insertion of the mouse mammary tumor virus. In *Xenopus* embryos ectopic expression of wnts can induce the formation of a secondary body axis, resulting in double-headed tadpoles, a phenomenon related to the capacity of the wnt pathway to induce dorsalization of the embryo. This *Xenopus* 'double axis assay' is frequently used to study the activity and hierarchy of wnt signaling components [2]. Homologs of wnts are also found in non-vertebrate species. In *Drosophila*, the homologous wingless pathway is involved in the establishment of segment polarity, wing formation and differentiation of the endoderm [1]. In *Caenorhabditis elegans* five wnt-like ligands have been identified by sequence. Best characterized so far is mom-2 ('more mesoderm'), which is involved in cell fate specification in the early embryo [3].

The wnt signal transduction pathway has been analyzed through a combination of genetic and biochemical approaches. It was shown to involve essentially similar components in vertebrates, *Drosophila* and *C. elegans* and a common picture of wnt signal-

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ing has emerged [4]. We will shortly summarize the main features of the pathway and give an overview of the molecular characteristics of the involved factors. The biochemical relationships of the components will then be discussed in more detail in the following sections.

Wnts mainly act on target cells in a paracrine fashion through members of the frizzled receptor family of seven transmembrane spanning proteins [5]. Binding of wnts occurs at an N-terminal cysteine-rich extracellular domain and leads to activation of the receptors by an unknown mechanism. It is still unclear how the signal is further transmitted from the receptor into the cytoplasm, but the C-terminal cytoplasmic domain and the cytoplasmic loops which connect the transmembrane domains are likely to interact with signaling molecules yet to be discovered. Frizzleds were first discovered in *Drosophila*, but a number of vertebrate homologs with distinct expression patterns have been described. The relationship between these and the different wnt ligands is only beginning to be analyzed.

A number of factors that affect the wnt-frizzled interaction have been identified (Fig. 1). The porcupine gene product is a transmembrane protein localized in the endoplasmic reticulum and is required for secretion of wingless in *Drosophila* [6]. Homologs of porcupine also exist in mammalian species, but their function with regard to wnt activity has not been reported. The GPI-linked heparan sulfate proteoglycan dally was shown to cooperate with frizzled, possibly by acting as a co-receptor for wingless [7,8]. Several other extracellular proteins have been shown to interact with wnts and prevent binding to frizzleds. Among these are cerberus [9], WIF-1 (wnt-interacting factor) [10], and secreted frzB receptors related to the cysteine-rich wnt binding domain of frizzleds (also called sFRPs, secreted frizzled-related proteins). These antagonize the wnt pathway by competing with frizzled receptors for wnts [11–13]. The secreted factor dickkopf-1 (Dkk-1) was also shown to block wnt activity, but it is not known whether it physically interacts with wnts [14,15].

Activated frizzled receptors induce the stabilization of the cytoplasmic component β-catenin by blocking the function of a multiprotein β-catenin destruction complex (Fig. 1; for references see the following sections). This complex consists of the scaffolding component axin, or the related conductin (also named axil). These bind to the tumor suppressor protein adenomatous polyposis coli (APC), the serine/threonine kinase GSK3β (glycogen synthase kinase 3β, zeste-white3/shaggy in *Drosophila*) and β-catenin through separate domains. In the absence of a wnt signal, GSK3β mediates the phosphorylation of β-catenin which is then recognized by the F-box protein slimb/βTrCP and the ubiquitination machinery, and finally degraded in proteasomes (Fig. 1, '−wnt'). In the presence of wnt, the cytoplasmic phosphoprotein dishevelled is activated and interferes with the β-catenin destruction complex. GSK3β activity is inhibited, and the phosphorylation of β-catenin is blocked. The activity of GSK3β may be suppressed by binding to GBP (GSK3β binding protein)/Frat1 (frequently rearranged in advanced T-cell lymphomas), which can also interact with dishevelled. The activity of the β-catenin destruction complex may also be regulated by dephosphorylation, as subunits of protein phosphatase 2A have been shown to bind both axin and APC. Hypophosphorylated β-catenin is no longer degraded and accumulates in the cytoplasm and the nucleus, where it associates with transcription factors of the LEF-1/TCF family (LEF-1, TCF-1, -3 and -4; below collectively referred to as TCF). The TCF/β-catenin complexes act as transcriptional activators of wnt target genes and transmit the wnt signal into the nucleus (Fig. 1).

The transcriptional activity of the TCF/β-catenin complexes can be modulated by different means (Fig. 1; for references see the following sections). A MAPK-related pathway involving TAK1 (TGFβ-activated kinase) and NLK (NEMO-like kinase) phosphorylates the TCF/β-catenin complex and interferes with its binding to DNA. Wnt target genes can also be silenced by the interaction of TCFs with transcriptional co-repressors such as TLE/groucho family members and CtBP. In *Drosophila* the histone acetyltransferase CBP/p300 was shown to acetylate TCF, which reduces the interaction with β-catenin. Furthermore β-catenin binds to other nuclear factors, such as the *Drosophila* zinc finger protein teashirt, or the TATA binding protein TBP.

Wnt signaling might also be influenced by the cadherin-based cell adhesion system. β-Catenin associates with the cytoplasmic domain of cadherins [16],

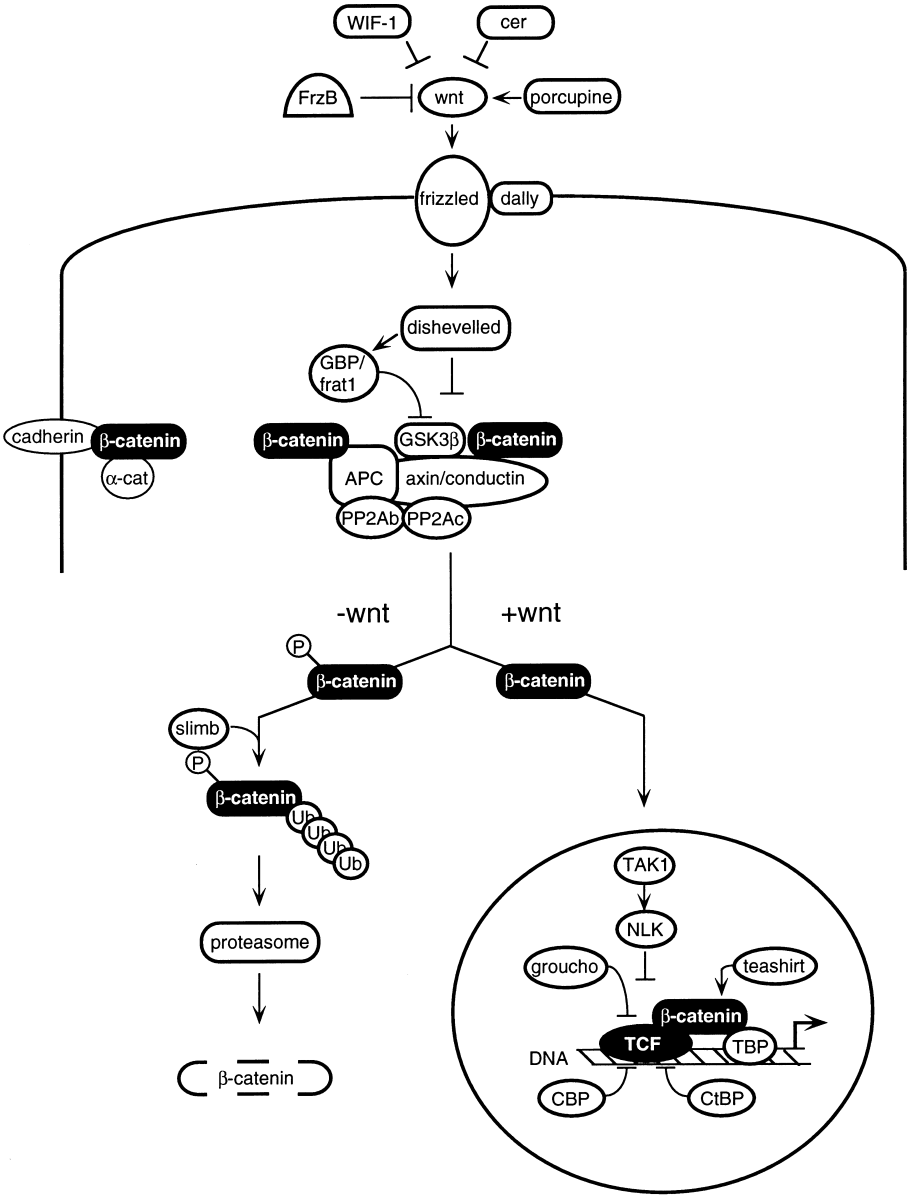

Fig. 1. Overview of the wnt pathway. Binding of wnts to frizzled receptors activates dishevelled which blocks the function of a complex assembled over the scaffold proteins axin or conductin. Note that axin and conductin are related proteins that can form similar complexes with APC, GSK3β, β-catenin, and PP2A and that APC can interact independently with β-catenin. In the absence of wnts (−wnt) the axin/conductin complexes promote phosphorylation of β-catenin by GSK3β. Phosphorylated β-catenin becomes multi-ubiquitinated (Ub) and subsequently degraded in proteasomes. In the presence of wnts phosphorylation and degradation of β-catenin is blocked which allows the association of β-catenin with TCF transcription factors (+wnt). The TCF/β-catenin complexes bind to DNA and activate wnt target genes. Additional regulatory factors that alter the activity of wnts or of downstream components and the complex of β-catenin with cadherins and α-catenin are also shown. For details refer to the text.

and binds the vinculin-related protein α-catenin, which in turn makes contact to actin filaments [17]. β-Catenin has a core domain of 12 so-called arm repeats [18] and binding of this region to cadherins and TCF factors is mutually exclusive [19]. Overexpression of the transmembrane and cytoplasmic domains of cadherins blocks axis formation in *Xenopus* embryos [20], and TCF/β-catenin mediated transcription in cell culture [19], presumably by depleting the cytoplasmic (signaling) pool of β-catenin. On the

other hand, studies in *Drosophila* show that the functions of β-catenin in cell adhesion and signaling can be completely separated: certain mutations of the β-catenin homolog armadillo only block cell adhesion, but do not affect wingless signaling, and armadillo mutants have been generated that lack signaling activity, but still function in cell adhesion [21]. Similarly, mutant β-catenin proteins defective in cell adhesion can still elicit a wnt signal in *Xenopus*, i.e. induce a secondary body axis [20]. The question remains whether cadherins are physiological regulators of wnt signaling. Conversely it has been suggested that wnt signaling regulates cadherin-based cell adhesion by altering the levels of β-catenin [22].

1.2. Wnt signaling in normal development

The in vivo function of wnt family members has been extensively investigated in *C. elegans*, *Drosophila*, zebrafish, *Xenopus*, chicken, and mice. We will here concentrate on the mouse system; several excellent reviews cover the results from other organisms [3,4,23–25]. Wnts are expressed in a tissue-specific manner, and mutant mice with deletion of certain wnt genes display strong phenotypes. For example, the lack of wnt-1 results in the deletion of part of the midbrain [26], and ablation of the wnt-4 and wnt-7a genes affects kidney and limb development, respectively [27,28]. Wnt-3 knockout mice are deficient in the formation of the anterior-posterior axis [29]. Interestingly, mutations in one of the mouse dishevelled homologs, Dvl-1, have no gross effects on embryonal development but lead to behavioral abnormalities in the adult [30]. Several studies suggest a role of the wnt pathway in epithelial-mesenchymal interactions during development. The knockout of the LEF-1 gene results in defects in the formation of teeth, hair follicles and the mammary gland, organs known to require inductive interactions of the epithelium with the underlying mesenchyme [31]. Transgenic mice that express LEF-1 in keratinocytes show alterations in hair follicle patterning [32], and transgenic overexpression of a dominant-active version of β-catenin in the skin induces the formation of additional hair follicles and results in hair follicle tumors [33]. The mutation of TCF-4 leads to abnormalities in the epithelium of the small intestine. In these mice, the stem cell compartment in the prospective crypts is missing, indicating that TCF-4/β-catenin signaling is required for continued proliferation of crypt cells [34]. The TCF-1 knockout is characterized by a block in the development of T-cells [35]. Somewhat astonishingly, the TCF null phenotypes described so far do not mimic known wnt mutations. However, mice double-deficient for LEF-1 and TCF-1 exhibit defects in the formation of paraxial mesoderm and develop additional neural tubes, a phenotype also seen in wnt3a-deficient mice [36]. This indicates that TCFs can have redundant roles in wnt signaling during mouse development.

1.3. Wnt signaling in cancer

Several lines of evidence demonstrate a role of the wnt signaling pathway and the TCF/β-catenin interaction in cancer. Certain wnts are found to be overexpressed in tumor samples (e.g. [37]) and a subset can transform cultured breast epithelial cells [38], but a causal role for wnts in the development of human cancer has not been established. In contrast, downstream components of the wnt pathway clearly play a fundamental role in tumor development. Increased nuclear staining of β-catenin and constitutive complexes with TCFs are frequently observed in tumor cell lines and tissue samples [39–42]. Stabilization of β-catenin in tumors results from mutations of the APC tumor suppressor, or of β-catenin itself [41,43]. Most human colorectal cancers are initiated by mutations of APC, which behaves as a classic tumor suppressor and shows loss of heterozygosity in the earliest recognizable benign tumors [44]. For this reason APC has been proposed to act as a gatekeeper for colonic epithelial cell proliferation. A causal relationship between the loss of APC and development of colorectal tumors has been confirmed in mice. Mice heterozygous for APC develop tumors in the intestinal tract, which show loss of heterozygosity for the APC gene [45,46], and conditional targeted mutation of APC in the mouse colon induces the formation of polyps, which progress to invasive carcinomas [47].

In colorectal tumors that lack mutations in APC, activating mutations of β-catenin have been identified. These mutations alter or delete critical serine and threonine phosphorylation sites in the amino-terminal domain of β-catenin that are required for

degradation [41]. While mutations of APC are mainly found in colorectal cancer, mutations of β-catenin have been found in a wide variety of tumor types, indicating a potent role for β-catenin as an oncogene in vivo (for review see [48]). On a cellular level, APC and β-catenin mutations correlate with increased amounts of cytosolic and nuclear β-catenin and transcriptionally active TCF/β-catenin complexes [41,49]. This results in the inappropriate activation of TCF target genes, including c-myc and cyclinD1 [50,51], which may ultimately lead to cancer. Furthermore, TCF/β-catenin complexes were shown to have transforming activity in some cell types [52,53]. In mice transgenic expression of a dominant-active β-catenin in the skin induces the formation of hair follicle tumors [33]. Interestingly, knockout mice deficient for TCF-1 were recently shown to develop adenomas in the gut and the mammary glands and additional mutation of APC increases the number of adenomas [54]. This indicates that TCF-1 acts as a repressor of oncogenic target genes in the absence of activated β-catenin (possibly through interaction with grouchos, see below), and that depletion of TCF-1 in the knockout animal results in the relieve of this repression and cellular transformation. Moreover, TCF-1 and APC may cooperate in tumor suppression [54].

2. The OFF state of the wnt pathway

Given the deleterious consequences of inappropriate activation of the wnt pathway, organisms have evolved several mechanisms to keep in check effectors of the pathway in the absence of a wnt signal. Free β-catenin levels are kept low by a multiprotein destruction complex that targets β-catenin to the proteasome. In addition TCF target genes can be silenced by transcriptional co-repressors that bind TCFs, or by posttranslational modifications of TCF/β-catenin complexes.

2.1. The β-catenin destruction complex

While β-catenin bound to cadherins is apparently quite stable, the cytosolic pool of β-catenin has a short half-life in the absence of a wnt signal. Its destruction is initiated by the action of a multiprotein complex which is assembled by the core components axin or conductin/axil. Axin or conductin can simultaneously bind to APC, β-catenin and GSK3β [55–60]. The complex promotes the GSK3β-dependent phosphorylation of β-catenin, which targets it for multi-ubiquitination and degradation by the proteasome.

Axin was identified as the gene mutated in the *Fused* mouse strains, of which certain alleles are recessive embryonic lethal and cause 'Siamese' axis duplications [61–63]. In *Xenopus* embryos, ectopic expression of axin prevents formation of the endogenous, and also of a wnt-induced secondary axis [63]. Mouse conductin and its rat ortholog axil were isolated as interaction partners of β-catenin and GSK3β, respectively [55,60]. Axin and conductin show 45% identity in their amino acid sequence [55,63,64] and appear to be functionally interchangeable. They consist of an amino-terminal 'regulator of G-protein signaling' (RGS) domain which binds to APC [55,56,64], separate binding domains for GSK3β and β-catenin in the center of the proteins

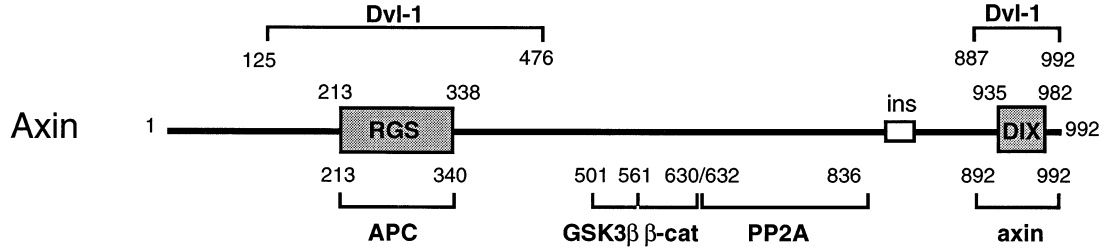

Fig. 2. Schematic representation of the structure of mouse axin. Amino acid numbering is according to [63]. The RGS domain, the DIX domain, and a 36 amino acid insert that is present in form 2 of axin are shown by boxes. Domains in axin interacting with APC [64], GSK3β [80], β-catenin [57], axin [65], and mouse Dishevelled-1 (Dvl-1 [109]) are indicated by brackets. Note that Dvl-1 can interact at two sites with axin [109].

[55,57,59], and a C-terminal (DIX) domain related to a segment of dishevelled, which mediates dimerization of axin (Fig. 2) [57,63,65,66]. When overexpressed in mammalian cells, axin and conductin promote the degradation of cytosolic β-catenin [55,56,64], prevent the wnt-induced accumulation of β-catenin [67], and transcription of a TCF-dependent reporter gene [59,65]. The apparent lack of enzymatic function and the direct interaction of axin family members with β-catenin, GSK3β and APC suggest that they function as scaffolds that enhance the activity of the complex by assembling the components in close proximity. Consistent with this view, axin stimulates the phosphorylation of β-catenin and APC by GSK3β [56,57,68].

APC appears to be a crucial component of the β-catenin destruction complex. In colon tumors, mutations of APC correlate with high levels of β-catenin and transcriptionally active TCF/β-catenin complexes. The re-introduction of wild-type APC into colorectal cancer cells reduces β-catenin levels [69], and antagonizes TCF/β-catenin-mediated transcription [49], confirming that APC negatively controls β-catenin stability. Several studies also suggest this function of APC during normal development. In *Drosophila*, inactivation of an APC homolog, which is mainly expressed in neuronal cells [70], results in TCF/β-catenin-mediated loss of photoreceptor cells by apoptosis [71]. A second *Drosophila* APC gene is expressed ubiquitously with particularly high levels in epithelial cells, and was named E-APC. Consistent with a role for E-APC in antagonizing the wnt pathway, dsRNA interference with E-APC results in phenotypes resembling ectopic wnt signaling. Interestingly, E-APC localizes to E-cadherin and β-catenin-containing adherens junctions and this localization pattern depends on GSK3β function [72]. While the above studies are compatible with the model that APC antagonizes wnt signaling by reducing β-catenin levels, not all experimental systems allow the same conclusion. In *C. elegans*, the null phenotype of an APC homolog mimics the loss of wnt or β-catenin function [73], and overexpression of APC in *Xenopus* embryos mimics wnt-induced axis duplication [74]. Both results suggest that APC may also be involved in the transmission of wnt signals.

APC contains heptad repeats at its N-terminus that mediate homo-oligomerization, and seven armadillo repeats of unknown function (Fig. 3). The C-terminal part contains a basic domain that binds microtubules, a region interacting with EB1, and a domain that binds the human homolog of the *Drosophila* discs-large protein (DLG) [75]. The central part of APC contains three 15 amino acid, and seven 20 amino acid repeats that both bind β-catenin [75]. Interspersed within the 20 amino acid repeat region of APC are three so-called SAMP repeats that have been shown to mediate the interaction with conductin and, by inference, axin [55]. In colorectal cancer cells, the 20 amino acid repeat region was shown to be sufficient to downregulate β-catenin [69]. In addition, the vast majority of mutations in APC occur in this region and result in C-terminally truncated APC proteins that in most cases still retain 20 amino acid repeats and bind to β-catenin, but lack all SAMP repeats. This suggests that the interaction with axin or conductin is required for APC to function as a tumor suppressor and that both proteins must cooperate to mediate the degradation of β-catenin. Indeed, knockout mice of APC that retain the N-terminal part including one SAMP repeat, but lack all the C-terminal domains do not show any signs of tumor formation [76]. In addition, expression of APC fragments containing the SAMP repeats results in the stabilization of β-catenin, presumably by interfering with the association of endogenous APC with conductin and axin [55]. Surprisingly, axin and conductin are capable of degrading β-catenin when overexpressed in cells that lack functional APC [55,56]. This indicates that axin and conductin can also function downstream from or independently of APC. On the other hand, conductin is expressed in colorectal cancer cells that lack functional APC (J. Behrens, unpublished observations), but is obviously not sufficient to control the high levels of β-catenin. It is likely that under physiological conditions the interaction of APC and axin/conductin is required for the degradation of β-catenin, while under conditions of overexpression axin or conductin are sufficient for degradation. It is not yet understood which essential function APC contributes to the destruction complex.

Another critical component of the β-catenin destruction complex is the serine/threonine kinase GSK3β that has multiple functions within the cell. In both *Drosophila* and *Xenopus*, interference with

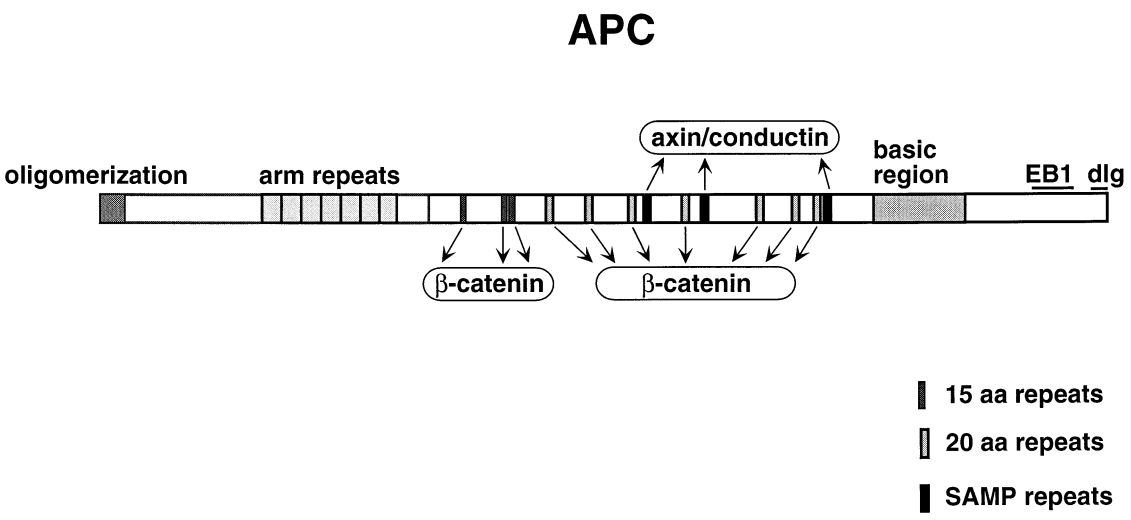

Fig. 3. Domain structure of APC and interaction with binding partners. The domains of APC are described in the text. The interaction of the 15 aa acid repeats and the 20 aa repeats with β-catenin and of the SAMP repeats with axin/conductin is indicated by arrows. The interaction of EB1 and dlg1 at the C-terminus of APC is also indicated (see text for details).

GSK3β function is sufficient to induce phenotypes characteristic for wnt signaling [77–79]. Conversely, ectopic overexpression of GSK3β prevents axis formation in *Xenopus* [78], and TCF-dependent reporter gene activation in mammalian cells [80]. Genetic evidence in *Drosophila* suggests that in the absence of a wnt signal, GSK3β antagonizes downstream elements of the wnt pathway by negatively regulating the levels of β-catenin [77]. GSK3β can phosphorylate specific serine and threonine residues in the N-terminus of β-catenin in vitro [79] and mutation of these sites results in the stabilization of β-catenin [41,79,81,82], suggesting that phosphorylation of β-catenin by GSK3β at these sites targets it for degradation. β-Catenin is generally not a good substrate for GSK3β in vitro, but is efficiently phosphorylated in the presence of axin [57].

Phosphorylated β-catenin is a target for multi-ubiquitination and degradation by the 26S proteasome (for review see [83]). Proteasome inhibitors stabilize β-catenin and promote the accumulation of β-catenin-ubiquitin complexes. Ubiquitination is prevented by wnt signaling, by treatment of cells with the GSK3β inhibitor LiCl, or by point mutations of the N-terminal phosphorylation sites in β-catenin [81,84]. The formation of ubiquitin-protein conjugates involves an enzymatic cascade of ubiquitin transfer reactions. In an ATP-dependent reaction, ubiquitin is first attached to a ubiquitin-activating enzyme (E1), then transferred to a ubiquitin-conjugating enzyme (E2), which cooperates with an E3 component to join ubiquitin to a lysine residue of the target protein. Multi-ubiquitinated proteins are recognized by the regulatory subunit of the proteasome and immediately degraded into short peptides. The substrate specificity of the ubiquitination machinery is determined by a large number of E3s [85]. Phosphorylated β-catenin is recognized by the E3 component slimb, which was identified in *Drosophila* [86]. The slimb phenotype mimics constitutive activation of the wnt pathway, indicating that slimb negatively regulates wnt signaling. Indeed, slimb mutant flies accumulate high levels of armadillo [86]. Slimb is highly homologous to *Xenopus* βTrCP and contains an F-box and seven WD40 repeats. In *Xenopus*, coexpression of βTrCP prevents formation of a wnt-induced secondary axis and a dominant-negative βTrCP lacking the F-box induces a secondary axis [87]. In mammalian cells, the overexpression of slimb/βTrCP enhances the ubiquitination of β-catenin, while a dominant-negative slimb/βTrCP lacking the F-box prevents ubiquitination and stabilizes β-catenin [88]. The WD40 repeat domain of slimb/βTrCP specifically recognizes phosphorylated β-catenin while the F-box recruits the general ubiquitination machinery into the complex [89]. The role of slimb/βTrCP in β-catenin degradation suggests that it might be a tumor suppressor; however, a prelimi-

nary analysis of four colon cancers wild-type for APC and β-catenin did not reveal mutations in slimb/βTrCP [90].

2.2. Transcriptional co-repressors silence TCF target genes

In the absence of wnt signals, tight control of β-catenin levels prevent its association with TCF and the transcription of wnt target genes. In addition, these genes can be actively repressed by the recruitment of transcriptional co-repressors to DNA-bound TCFs (for review see [25,91]).

TCFs bind to members of the TLE/groucho family of co-repressors [92–94], and coexpression of TLE/groucho with TCF inhibits transcription of a TCF-dependent reporter gene, even in the presence of β-catenin [92,93]. In *Xenopus*, TCF-3 can also mediate repression by binding to the transcriptional co-repressor CtBP (C-terminal binding protein) [95]. Genetic experiments in *C. elegans* and *Drosophila* suggest that the interaction of TCFs with co-repressors is of physiological importance. In *C. elegans*, loss-of-function mutations in POP-1, a TCF homolog, show a phenotype resembling constitutive wnt signaling, suggesting that POP-1 acts as a transcriptional repressor [73,96]. That TCFs mediate repression is further indicated by the finding that in *Drosophila*, a reduction in gene dosage of TCF, or loss of function of groucho, suppresses the phenotype of wingless or armadillo mutations, presumably by de-repressing wingless target genes [94].

2.3. Posttranslational modifications antagonize TCF activity

In *Drosophila* TCF was shown to interact with CREB binding protein (CBP/p300) [97]. CBP has been shown to act as a transcriptional co-activator in a variety of settings, in which it presumably alters chromatin structure by virtue of its histone acetylase activity. Surprisingly, in the context of TCF, CBP acts as a transcriptional co-repressor. As expected for a repressor, loss-of-function mutations of CBP suppress wingless and armadillo phenotypes. The mechanism by which CBP inhibits TCF function is not entirely clear, but CBP has been shown to acetylate a specific lysine residue in the armadillo binding domain of TCF. This modification reduces the interaction of TCF with armadillo [97].

TCF/β-catenin complexes can be phosphorylated by a MAPK-related pathway, which prevents their binding to DNA. In *C. elegans*, homologs of TAK1 (TGFβ activated kinase-1), a MAP-kinase-kinase-kinase family member, and of NLK (NEMO-like kinase), a member of the MAP-kinase family [98,99], were shown to be required for the downregulation of TCF activity. In *Xenopus*, ectopic expression of NLK blocks formation of the endogenous axis, and prevents the formation of a β-catenin-induced secondary axis [100]. In mammalian cells NLK also inhibits β-catenin-stimulated TCF reporter gene activity [100]. At least in *C. elegans*, β-catenin interacts directly with NLK and can stimulate its kinase activity [99]. This leads to the phosphorylation of TCF and its relocalization from the nucleus to the cytoplasm [99]. In bandshift assays, NLK reduces binding of TCF/β-catenin complexes to DNA. NLK is apparently activated by the kinase TAK1 and its binding protein TAB1. Coexpression of TAB1 and TAK1 stimulates the kinase activity of NLK and inhibits the transcriptional activation of a TCF reporter gene by β-catenin [100]. Genetic evidence in *C. elegans* also suggests that TAB1 is involved in the inhibition of TCF activity upstream of TAK1 [98]. The physiological role of this pathway is best documented in *C. elegans*, where it cooperates with wnt signaling in the establishment of posterior cell fates [98,99]. During wnt signaling in *C. elegans* β-catenin cooperates with the TAK1/NLK kinases to downregulate TCFs activity as a repressor. It will be of interest to further analyze the physiological role of this pathway in mammalian development and cancer.

3. The ON state of the wnt pathway

Central to the transmission of the wnt signal is the inhibition of GSK3β that results in the functional inactivation of the β-catenin destruction complex. The subsequent activation of wnt target genes is then mediated by TCF/β-catenin complexes.

3.1. Dishevelled

Genetic epistasis experiments in *Drosophila* suggest

that the inhibition of GSK3β function is mediated by the cytoplasmic protein dishevelled. Ectopic expression of dishevelled mimics wnt signals in *Drosophila* and *Xenopus* systems [101,102]. In mammalian cell culture, overexpression of dishevelled inhibits the catalytic activity of GSK3β [103] and stimulates TCF-dependent reporter gene activity [80]. While dishevelled appears to lack enzymatic function, it contains potential interaction motifs found in signal transduction components of other pathways (for review see [104]), namely an N-terminal DIX domain (found in dishevelled and axin), a central PDZ domain, and a C-terminal DEP domain (dishevelled, egl-10 and pleckstrin). Both the DIX and PDZ domains were shown to be required for the stabilization of β-catenin in *Drosophila* cell lines [102], while the DEP domain appears to be necessary for an alternative wnt-dependent 'planar cell polarity' pathway involving Rho and Jnk [105]. How dishevelled is activated by frizzled is unknown, but dishevelled is hyperphosphorylated upon wnt stimulation. Hyperphosphorylation correlates with the stabilization of β-catenin. However, the overexpression of *Drosophila* frizzled2 in the absence of ligands also results in hyperphosphorylation of dishevelled, but does not affect β-catenin levels [102,106]. Maybe several kinases are involved in the activation of dishevelled.

3.2. Dishevelled interacts with casein kinase Iε (CKIε)

CKIε can bind to the PDZ domain of dishevelled and the overexpression of CKIε results in increased phosphorylation of dishevelled. In cultured cells, CKIε can be coprecipitated with dishevelled, axin, and GSK3β. The CKI family consists of seven family members in mammals and the δ and ε isoforms are distinguished by an additional C-terminal domain not found in the other members. This C-terminal domain appears to be important in wnt signaling, as CKIε lacking the C-terminal domain does not coprecipitate with components of the destruction complex [107,108]

Overexpression of CKIε mimics wnt signaling in a variety of assays. In *Xenopus* embryos, ventral injection of CKIε induces a secondary axis, while mutants lacking kinase activity or the C-terminal domain do not. CKIε, like wnt, also rescues UV ventralized embryos. CKIε induces wnt target genes, including siamois and nodal-related 3, and low levels of both Xwnt-8 and CKIε synergistically activate these genes. In mammalian cells CKIε also activates a TCF-dependent reporter gene. These activities of CKI correlate with increased β-catenin levels [107,108].

Interference with CKIε is also consistent with a role in the wnt pathway. In *Xenopus*, dominant negative forms, as well as pharmacological inhibitors, prevent the formation of a wnt-induced secondary axis. Inhibition of CKI also blocks dishevelled-induced secondary axis formation, but does not inhibit the effect of injected β-catenin. In *C. elegans*, RNAi with the close homolog kin-19 mimics loss of function of wnt. In mammalian cells, a kinase-dead CKIε, or CKIε antisense oligonucleotides inhibit the wnt-induced TCF-dependent reporter gene activity [107,108]. Further epistasis experiments place CKIε downstream of wnt, frizzled and dishevelled and upstream of GSK3β and β-catenin, suggesting that CKIε is involved in the dishevelled-mediated inhibition of the β-catenin destruction complex. Casein kinase-2 (CK2) has also been shown to phosphorylate dishevelled, but is not sufficient for the activation of dishevelled and downstream events [106].

3.3. Dishevelled interacts with axin

Two domains of dishevelled required for transmission of the wnt signal have been shown to directly interact with axin (Fig. 2) [67,109]. The N-terminal 200 amino acids (aa) of dishevelled, including the DIX domain, interact with the C-terminal 105 aa of axin that also include its DIX domain. In addition, the PDZ domain of dishevelled interacts with the amino-terminal region of axin [109]. A C-terminal fragment of axin containing little more than the DIX domain inhibits the wnt-induced activation of a TCF-dependent reporter, probably by interfering with the axin/dishevelled interaction [109]. This suggests that dishevelled blocks axin function by direct interaction. Indeed, high concentrations of recombinant dishevelled can inhibit the axin-mediated phosphorylation of β-catenin by GSK3β in vitro [67]. The mechanism of inhibition is not understood, but it is dependent on the PDZ domain of dishevelled. Further, dishevelled does not compete with GSK3β for

binding to axin [67] nor does it inhibit the catalytic activity of GSK3β toward a synthetic peptide substrate [110]. It is possible that dishevelled alters the conformation of axin such that the accessibility of β-catenin for GSK3β is reduced.

3.4. GBP/Frat1 can bind and inhibit GSK3β

An alternative mechanism for the inactivation of GSK3β by dishevelled was suggested by the isolation of the *Xenopus* GSK3β binding protein GBP. Overexpression of GBP blocks the activity of GSK3β in *Xenopus* embryos and leads to axis duplication, while antisense interference with endogenous GBP prevents axis formation [111]. GBP is closely related to a mouse protooncogene Frat1 (frequently rearranged in advanced T-cell lymphoma), which was independently isolated as an oncogene in lymphocytes [112]. In mammalian cells the overexpression of Frat1 can activate a TCF reporter gene [109]. While the C-terminus of Frat1 binds to GSK3β, the N-terminus of Frat1 has been shown to bind the PDZ domain of dishevelled. Overexpression of a Frat1 fragment including the dishevelled binding domain inhibits wnt-induced TCF reporter gene activity, suggesting that the dishevelled/Frat1 interaction is necessary for wnt signaling. Since Frat1 is also found in a complex with axin, it might be involved in the transmission of the signal from dishevelled to the β-catenin destruction complex. In the presence of wnt, Frat1 and GSK3β have been observed to dissociate from dishevelled and axin [109].

3.5. Role of PKC in the inactivation of GSK3β

Wnt-mediated inactivation of GSK3β seems to require the activity of a protein kinase C (PKC). Activation of PKC with TPA leads to inactivation of GSK3β in the absence of a wnt signal and down-regulation of PKC by prolonged treatment with TPA prevents wnt-mediated inactivation of GSK3β [113]. In vitro, several PKC isoforms can phosphorylate and inactivate GSK3β [114]. Overexpression of PKCβII in the colonic epithelium of mice results in hyperproliferation of intestinal epithelial cells and increased sensitivity to carcinogen-induced colon cancer, which could be due to aberrant activation of the wnt pathway. Consistent with this idea, the colonic epithelium of PKCbII transgenic mice shows reduced GSK3β activity and elevated β-catenin levels [115].

3.6. Dissociation of the β-catenin destruction complex

Once the action of GSK3β is restrained, β-catenin becomes dephosphorylated and invisible for slimb/βTrCP and the ubiquitination machinery. However, to interact with downstream components of the wnt pathway, it must also be released from axin/conductin and APC. Apparently, the inhibition of GSK3β has fatal consequences for the destruction complex that promote the release of β-catenin.

In the absence of wnt signals, GSK3β not only phosphorylates β-catenin, but also axin [57,110, 116]. Upon wnt stimulation or inhibition of GSK3β with LiCl, axin is dephosphorylated [110,116]. Dephosphorylation of axin may cause the release of β-catenin from the destruction complex by two means. First, the affinity of hypophosphorylated axin toward β-catenin is reduced: beads coated with β-catenin precipitate only the phosphorylated form of axin from cell extracts. Conversely, immobilized recombinant axin precipitates more cellular β-catenin, when axin is first in vitro phosphorylated by GSK3β [116]. Second, the hypophosphorylated axin protein is unstable: prolonged stimulation with wnt, or treatment with LiCl, decreases the cellular levels of axin protein, while the phosphatase inhibitor okadaic acid stabilizes axin [110,116].

Axin also stimulates the phosphorylation of APC by GSK3β [56,68], and GSK3β has been shown to phosphorylate APC within the central 20 aa repeat region [117]. All seven 20 aa repeats contain an SXXXS(P) motif, which is likely to be the target for GSK3β. The 20 aa repeats bind β-catenin, and phosphorylation of this region by GSK3β in vitro has been shown to enhance the binding to β-catenin [117]. As suggested for axin, wnt signals might result in the dephosphorylation of APC resulting in its dissociation from β-catenin. There is also some evidence to suggest that catalytically inactive GSK3β is also excluded from the destruction complex. Axin, APC and β-catenin coprecipitate with wild-type, but not with kinase-dead GSK3β [57,117]. Taken together,

these data suggest that the integrity of the destruction complex depends on GSK3β-dependent phosphorylation events and that inhibition of GSK3β function causes a dissociation of the destruction complex by decreasing the affinity of the components for one another.

It is likely that a rapid response to wnt signals requires the action of phosphatases that reverse the GSK3β-mediated phosphorylation steps. The catalytic subunit of protein phosphatase 2A (PP2A) can bind to and dephosphorylate axin [65,116] and the phosphatase inhibitor okadaic acid prevents wnt-induced dephosphorylation of axin [116]. The PP2A regulatory subunit B56 binds to APC, and when overexpressed, reduces β-catenin levels and inhibits target gene transcription [118]. It is presently unclear whether overexpression of B56 stimulates or prevents the dephosphorylation of signaling components. It is intriguing that mutations of the PP2A A subunit have been identified in colon and lung cancers [119].

3.7. Nuclear translocation of β-catenin

Inactivation of the β-catenin destruction complex leads to the cytosolic accumulation and subsequent nuclear translocation of β-catenin. β-Catenin lacks an obvious nuclear localization sequence (NLS) for importin α/β receptors, and two distinct mechanisms for the translocation process have been proposed. First, free β-catenin might 'piggy-back' into the nucleus bound to newly synthesized or shuttling TCFs [120–123]. Alternatively, β-catenin may enter the nucleus independently of TCFs. Mutant forms of β-catenin unable to bind to TCF can still translocate to the nucleus [21,124]. Moreover, β-catenin was able to enter the nucleus in an in vitro nuclear import assay. Translocation of β-catenin was independent of importins, mimicking the behavior of importin β in such assays [125,126]. It is unclear whether the nuclear translocation of β-catenin is a default consequence of its cytosolic accumulation, or further regulated by wnt signals. Of interest in this respect is the interaction of β-catenin with the presenilins, which are proteins altered in Alzheimer's disease. It has been suggested that presenilins are involved in the nuclear translocation of β-catenin upon wnt signaling [127].

3.8. Activation of transcription

TCFs are sequence-specific DNA binding proteins, but apparently have no classical transactivation domain on their own. In immune cells LEF-1 appears to act as context-dependent transcriptional activator that requires cooperativity with neighboring transcription factors [128,129]. Apparently two distinct mechanisms are involved in the activation of wnt target genes by TCF/β-catenin complexes and there is evidence that both mechanisms may operate during wnt signaling [25]. First, TCFs can behave as active repressors of gene expression and wnt signaling relieves this repression. Second, TCFs can behave as 'silent' transcription factors that activate gene transcription upon binding to β-catenin. Collectively the data suggest that TCFs mediate repression of target genes in the absence of wnt signaling, while acting as transcriptional activators in the presence of wnt.

There is genetic evidence that wnt signaling results in de-repression of TCF-mediated transcription. In transgenic flies, the addition of TCF binding sites to a minimal synthetic promoter stimulates expression in cells that receive a wnt signal, but reduces expression in cells that do not [130]. In *Xenopus*, TCF/β-catenin complexes are active only in the dorsal part of the embryo and locally activate siamois expression. Elimination of the three TCF binding sites in the siamois promoter results in reduced dorsal, but increased ventral expression of the gene [131].

As described above, TCFs may repress gene expression by binding to transcriptional co-repressors such as TLE/groucho or CtBP. It is not clear how β-catenin interferes with TLE/groucho-mediated repression of target genes. The binding sites in TCFs of TLE/groucho and β-catenin do not overlap which rules out direct competition of both factors [132]. As discussed above, CBP antagonizes wingless signaling by acetylating dTCF at its armadillo binding domain. Binding of armadillo to dTCF has been shown to reduce the ability of CBP to catalyze this reaction, perhaps by steric interference [97].

If TCFs acted solely as repressors, their loss-of-function phenotypes should mimic activated wnt signaling; however, loss of TCF function in *Drosophila* and *Xenopus* mimics loss of function of wnt or β-cat-

enin [120–122,130,133,134]. This suggests a positive role for TCFs as transcriptional activators during wnt signaling. Transcriptional activation depends on the binding of TCF to β-catenin. Reporter constructs containing TCF binding sites could be activated by coexpression of TCF and β-catenin, but not by TCF alone [122]. Genetic experiments show that the C-terminal domain of armadillo is indispensable for wingless signaling [77] and both C- and N-terminal regions of β-catenin have been shown to provide transactivation domains. For instance, fusion proteins of the N- or C-terminal domains of β-catenin with the Gal4 DNA binding domains activate Gal4 responsive promoter constructs [133,135,136]. Fusion proteins of LEF-1 with the C-terminal domain of β-catenin, or with the potent VP16 transactivation domain activate LEF-1 dependent promoters to a similar extent [137]. Moreover both fusion proteins induce axis duplication in *Xenopus* indicating that the transactivation function of β-catenin is sufficient for wnt signaling in this particular experimental setting. Similarly, cell transformation of chicken embryo fibroblasts could be achieved by expression of chimeras of LEF-1 and various heterologous transactivation domains [52]. TCF/β-catenin complexes can thus be regarded as bipartite transcription factors in which the DNA binding and transactivation functions are contributed by two separate proteins.

How the transactivation domains of β-catenin activate transcription is presently unclear but associations of β-catenin with the TATA binding protein (TBP) have been shown [136]. In addition β-catenin might contact TBP indirectly via binding to Pontin52 [138]. This indicates that TCF/β-catenin complexes directly recruit components of the basal transcriptional machinery to wnt target genes. Interestingly, expression of LEF-1 and β-catenin in normal T cells was not sufficient to activate reporter gene transcription suggesting that LEF-1/β-catenin require additional factors not present in these cells [124].

3.9. Wnt target genes

Several genes whose expression is regulated by TCF/β-catenin complexes have been described. The c-myc gene was shown to be downregulated by conditional expression of APC in a colorectal cancer cell line. The c-myc promoter contains TCF binding sites which mediate transcriptional activation by TCF/β-catenin as well as repression by APC in reporter gene assays. A dominant-negative version of TCF-4 reduced endogenous levels of c-myc, indicating that TCF/β-catenin complexes are essential regulators of the c-myc gene in colorectal cancer cells [50,139]. The cyclinD1 promoter also contains TCF binding sites and is activated by β-catenin; importantly, dominant-negative TCF causes arrest of colon cancer cells in the G_1 phase of the cell cycle, which can be rescued by expression of cyclinD1 [51]. Further targets for β-catenin signaling with relevance for tumor development have been identified, such as components of the AP-1 complex and the extracellular matrix protease matrilysin [140,141]. Genes involved in developmental aspects of wnt signaling, such as siamois [131], ultrabithorax [130], nodal-related 3 [142], and twin [143] are direct targets of TCF/β-catenin complexes. The promoters of these genes contain TCF binding sites which are essential for transcriptional activation as demonstrated by mutational analysis. Given the multiple activities of wnts it is certain that the list of target genes will increase in the future.

4. Conclusions and perspectives

Significant progress has been made over the years in understanding the biochemical mechanisms that regulate the wnt pathway. This has been paralleled by the analysis of the in vivo function of key signaling components. Important questions remain to be answered. For instance, our knowledge of the repertoire of direct target genes of wnt signaling is still limited. Given the importance of the pathway in both normal development and cancer it is likely that efforts will be made to discover new TCF/β-catenin target genes. As seen in other fields of signal transduction research, the wnt cascade might be part of a larger network of signaling systems. Several components such as GSK3β and factors of the ubiquitination machinery appear to be regulated by stimuli different from wnt and might represent entry points for cross-talk to other systems. The interest in the wnt signaling pathway has been nourished by the finding of its oncogenic relevance. It is obvious that interference with the pathway in tumors could be of therapeutic value in anticancer treatment.

Drugs might be searched for that block the interaction of TCFs and β-catenin, or prevent transcriptional activation by the complex. In addition, when more is known about the biochemical mechanisms that regulate β-catenin stability, it might become possible to devise strategies that enhance the degradation of β-catenin and thereby block its accumulation in tumor cells.

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